Regulation of Circadian Rhythms and Flowering

BACKGROUND OF THE INVENTION

The present application is based on United States Provisional Application 60/094,072, entitled "A Method or Regulating the Function of a Gene Involved in Circadian Rhythms and Flowering" filed on July 24, 1998, and claims priority from that application. Research leading to this invention was supported by NIH grant R01-GM-23167 and the Government may have rights in this patent.

1. Field of the Invention

The present application concerns molecular biology and more specifically the molecular components of the "clock" that times biological processes in green land plants.

2. Introduction and Related Art

Endogenous circadian rhythms exist in a wide variety of organisms both multicellular plants and animals as well as microorganisms. Circadian clocks regulating these rhythms consist of input pathways, a central oscillator and output pathways (14, 26, 48). Oscillators are thought to generate rhythms by a transcription-translation negative feedback loop (65, 16, 15, 64, 46). Studies in cyanobacteria, *Neurospora*, *Drosophila* and mouse have found that both positive and negative elements that activate and inhibit the transcription of clock genes are required to maintain the feedback loop (16, 15, 64, 46). In addition, posttranscriptional and posttranslational regulation play an important role in circadian clocks in *Drosophila* and *Neurospora* (65, 51, 57). Input pathways from

environmental cues such as light and temperature can entrain the oscillator, and it, in turn, regulates specific cellular events such as expression of clock-controlled genes (14, 26, 48). Until recently, little was known about circadian clocks in plants (33). In *Arabidopsis thaliana*, the *toc1* mutant affects the period of many circadian rhythms (37, 52). Although the corresponding gene has not yet been cloned, it is thought that *TOC1* encodes a component of the oscillator. The *ELF3* gene has been proposed to act in the input pathway (23).

The phytochromes, a class of plant photoreceptors that has been extensively studied (44), regulate the expression of many genes, including the *Lhcb* genes which encode the chlorophyll a/b-proteins of photosystem II (59). A promoter region of the *Lhcb1*3* gene of *Arabidopsis thaliana* that is essential for its regulation by phytochrome was identified (56 ,27), and the *CCA1* gene, whose product specifically interacts with this promoter region, was cloned (63). The *CCA1* gene forms the subject of U.S. Patent Application 08/843572, filed on April 18, 1997, which is incorporated herein by reference. The motif to which CCA1 binds is highly conserved in promoters of *Lhcb* genes from many species. Transgenic *Arabidopsis* plants expressing antisense CCA1 RNA showed reduced phytochrome induction of the endogenous *Lhcb1*3* gene in etiolated seedlings. Furthermore, the increase in CCA1 mRNA in response to light preceded the increase in *Lhcb1*3* mRNA (63). These data showed that CCA1 is a downstream component of the phytochrome signal transduction pathway leading to increased transcription of the *Lhcb1*3* gene in *Arabidopsis*.

Expression of the *Lhcb* genes is also regulated by circadian rhythms (36). Characterization of CCA1 has shown that it is also involved in the circadian regulation of the *Lhcb1*1* gene and in the control of other physiological rhythms, such as timing of flowering. CCA1 mRNA and protein levels themselves exhibit circadian oscillations, and overexpression of CCA1 repressed the expression of the endogenous *CCA1* gene. Our earlier experimental results have demonstrated that the

function of CCA1 is closely associated with the circadian oscillator itself (62). *LHY*, has also been identified as a potential clock genes (49). Constitutive expression of *CCA1* was shown to abolish several distinct circadian rhythms and suppress its own expression as well as the rhythmic expression of *LHY* (61, 62). Lack of CCA1 in a T-DNA insertion mutant line shortened the periods of *LHY* and other clock-controlled genes (19). Overexpression of *LHY* also caused photoperiod insensitivity, arrhythmic expression of clock-controlled genes, and reduction of its own expression (49). These data suggest that both *CCA1* and *LHY* may encode components of regulatory negative feedback loops closely associated with the central oscillator. The *ESD4* (*Early Short Days 4*) gene of *Arabidopsis* is the subject of a patent publication (WO 98/56918) and has also been reported to alter responses to photoperiod.

SUMMARY OF THE INVENTION

To understand how CCA1 may function in the phytochrome signal transduction pathway and in the regulation of circadian rhythms, a yeast two-hybrid system was used to identify proteins that can interact with the CCA1 protein. A gene designated *CKB3* whose product interacts specifically with CCA1 has been identified through use of the yeast two-hybrid system. CKB3 is a structural and functional homologue of the regulatory (β) subunit of protein kinase CK2 in *Arabidopsis*. CK2 is a Ser/Thr kinase that is expressed ubiquitously and consists of two catalytic α- and two regulatory β-subunits. CKB3 and other β-subunits of CK2 interact specifically with CCA1 both in the yeast two-hybrid system and *in vitro*. Recombinant CK2 can phosphorylate CCA1 *in vitro*. Furthermore, *Arabidopsis* plant extracts contain a CK2-like activity that affects the formation of a DNA-protein complex containing CCA1. These results suggest that CK2 can modulate CCA1 activity, and that CK2 may play a role in the regulation of the circadian clock (55, 26, 48).

Recombinant plants that overexpress *CKB3* were constructed. Overexpression of *CKB3* resulted in increased CK2 activity and resulted in shorter periods of rhythmic expression of *CCA1* and *LHY*, as well as of four other circadian clock-controlled genes. This resulted a significant shortening of time to flowering under short-day conditions. This change in flowering time was not accompanied by significant phenotypic changes in morphology. Alteration of CK2 activity, particularly through the overexpression of the CK β-subunits represents a new and effective way of modulating flowering time in plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. illustrates the structure of *Arabidopsis* CKB3 and its homology with *Arabidopsis* CKB1 and CKB2. Fig. 1A shows cDNA sequence and corresponding deduced amino acid sequence of *Arabidopsis* CKB3. Clone 106 cDNA sequence is underlined. An upstream in-frame stop codon is shown in bold type. Nucleotide numbers are on the left and amino acid numbers on the right. Fig. 1B) illustrates alignment of predicted amino acid sequences of *Arabidopsis* CKB1, CKB2, and CKB3. Vertical lines indicate identical amino acid residues and conservative amino acid replacements are indicated by single and double dots. Dashes represent gaps introduced to give maximal identity.

Fig. 2 shows SEQ. I.D. No. 1, the cDNA sequence of CKB3 as shown in Fig. 1.

Fig3 shows SEQ I.D. No. 2, the amino acid sequence of CKB3 as shown in Fig. 1.

Fig. 4. is a photograph of culture plates demonstrating compensation of the *cka1-Δ1 cka2-8* temperature-sensitive mutation by *Arabidopsis CKB3* cDNA. YDH8 (*cka1-Δ1, cka2-8*) was transformed with yeast expression vectors, and incubated at 25°C or 35.5°C. Transformants harboring pKT10 vector only, pJCR14 carrying the

S. cerevisiae CKB2 gene, and pKT-CKB3 carrying the *Arabidopsis CKB3* gene are shown.

Fig. 5. shows the interactions of CCA1 with CK2 subunits. Fig. 5A shows interactions of CCA1 and CKB1, CKB2, and CKB3 in yeast. Each panel shows triplicate patches of yeast expressing GAL4-DB (left lines), GAL4-DB-SNF1 (middle lines), or GAL4-DB-CCA1 (right lines) transformed with GAL4-AD-CKB1 (top rows), GAL4-AD-CKB2 (middle rows), or GAL4-AD-CKB3 (bottom rows). Top left panel (+His), control plate containing histidine; top right panel (-His), plate lacking histidine (selective for the *HIS3* reporter gene expression); bottom panel (β-gal), β-galactosidase assay performed on a filter. Dark color shows β-galactosidase activity accumulated after a 3 hr incubation with substrate. Fig. 5B is an autoradiograph of SDS-PAGE analysis showing *in vitro* interactions between CKB1, CKB3, CKA1 and CCA1. GST-CCA1 or GST immobilized on glutathione-agarose beads was mixed with ^{35}S-labeled CKB1, CKB3, CKA1 or GBF4. The amount of proteins bound to GST (lanes 4-6, 11), or GST-CCA1 (lanes 7-9, 12) is shown. Lanes 1-3, and 10 represent 5% of the ^{35}S-labeled proteins used.

Fig. 6. illustrates that CK2 β-subunits enhance the binding of CCA1 to the *Lhcb1*3* promoter. Autoradiographs of the EMSA are shown. The ^{32}P-labeled A2 fragment was incubated with 0.5 ng of CCA1 in the presence of increasing amounts of His-tagged CKB3 (left panel) or CKB1 (right panel) (lanes 4 and 11, 1 ng; lanes 5 and 12, 2 ng; lanes 6 and 13, 5 ng; lanes 7 and 14, 10 ng). Lanes 1 and 8, probe alone; lanes 2 and 9, the A2 fragment with 50 ng of CKB3 and CKB1; lanes 3 and 10, the A2 fragment with 0.5 ng of CCA1 and 50 ng of BSA.

Fig. 7. shows phosphorylation of CCA1 by CK2 *in vitro*. Fig. 7A is an autoradiograph of SDS-PAGE analysis which shows that CKA1 can phosphorylate CCA1. GST (lane 1) or GST-CCA1 (lanes 2 and 3) incubated with 280 ng of CKA1 (lanes 1 and 3) or without CKA1 (lane 2) in the presence of [γ-^{32}P]ATP. Fig. 7B

shows that CK2 β-subunits enhance the phosphorylation of CCA1 by CKA1. GST-CCA1 (lanes 1-7) or GST alone (lanes 8 and 9) was incubated with 14 ng of CKA1 (lanes 2-9) or without CKA1 (lane 1) in the presence of [γ-^{32}P]ATP (lanes 1-4, 8, and 9) or [γ-^{32}P]GTP (lanes 5-7). Lanes 2 and 5, CKA1 alone; lanes 3, 6, and 8, with 35 ng of CKB1; lanes 4, 7, and 9, with 35 ng of CKB3. Arrows in both panels indicate the position of the full-length GST-CCA1 protein. Other bands might be degradation products of GST-CCA1.

Fig. 8. demonstrates that *Arabidopsis* plants contain a CK2-like activity that phosphorylates CCA1 *in vitro*. GST (lanes 1 and 4) or GST-CCA1 (lanes 2, 3, 5-7) were incubated with 160 μg of WCE in the absence (lanes 1, 2, 4, and 5) or presence (lane 3) of 10 mM 2,3-diphosphoglycerate (D. G.), with recombinant CK2 (rCK2) in the absence (lane 6) or presence of 10 mM 2,3-diphosphoglycerate (lane 7) together with [γ-^{32}P]ATP (lanes 1-3, 6, and 7) or [γ-^{32}P]GTP (lanes 4 and 5). The arrow indicates the position of the full-length GST-CCA1 protein.

Fig. 9. shows that CK2 phosphorylation is required for the formation of a DNA-protein complex containing CCA1. Fig. 9A shows that phosphatase treatment abolishes CCA1 binding. WCE were incubated in the absence (lane 1) or presence (lane 2) of λ protein phosphatase (200 units added to 20 μl) for 30 min at 30 °C. Fig. 9B shows that the CCA1 containing complex is more abundant in plants overexpressing CCA1 than in wild type. WCE from wild type (WT) and a CCA1-ox line (CCA1-ox) (7) grown in 12:12 photoperiods were used in the EMSA. Fig. 9C shows that inhibition of CK2 activity in plant extracts abolishes CCA1 binding. WCE were incubated with the indicated inhibitor for 45 min at 30 °C. Lane 1, no inhibitors; lane 2, 5 mM 2,3-diphosphoglycerate (D.G.); lane 3, 100 μM quercetin. Arrows indicate the position of the major DNA-protein complex containing CCA1.

Fig. 10. shows that overexpression of *CKB3* increases CK2 activity. Fig. 10A shows gel results of a quantitative RT-PCR analysis of *CKB3* and *UBQ10*

transcript levels in wild-type (WT) and independent homozygous transgenic lines (ox18 and ox41) after 14 days growth under L:D 16:8 photoperiods. PCR products were detected by Southern blotting using ^{32}P-labeled probes. The *UBQ10* transcript levels were used as an internal control. Fig. 10B shows a Western blot analysis of CKB3 levels in transgenic lines grown as in Fig. 10A. The c-myc-tagged CKB3 protein (arrow) was detected with monoclonal anti-c-myc antibody. Other bands are non-specific. Molecular size markers are given to the left in kiloDaltons. Fig. 10C shows a graph of CK2 activity in plant extracts prepared from wild-type and *CKB3*-ox lines. Plants were grown for 20 days under continuous white light. The data shown are means of two independent experiments for each line with the range of the measurements indicated.

Fig. 11. demonstrates that overexpression of *CKB3* shortens periods of *CCA1* and *LHY* circadian oscillations. Fig. 11A shows gel electrophoresis demonstrating Circadian oscillation of *CCA1* expression in wild-type and *CKB3*-ox (line ox18) plants. Plants were grown for 12 days in L:D 12:12 photoperiods then transferred to continuous light after light-on of day 13. After 16 h, tissue was collected every 4 h. Total RNA was isolated and analyzed for *CCA1* transcripts by Northern blot analysis with ^{32}P-labeled probes. The *UBQ10* RNA levels were used as an internal control for quantitation. A representative autoradiogram is shown in the upper panel. The lower panel shows the quantitation of one RNA blot. Values were normalized to the lowest value of the wild-type samples. Closed squares (solid line), wild-type; circles, *CKB3*-ox. The bar represents the subjective light conditions for the wild-type plants. Experiments were performed three times with similar results. The same results were also obtained in another *CKB3*-ox line (ox41). Fig. 11B illustrates gels showing circadian oscillation of *LHY* in wild-type and *CKB3*-ox plants. *LHY* transcripts in the same RNA samples were analyzed as in Fig. 11A. Fig. 11C illustrates CCA1 protein levels in wild-type and CKB3-ox (line ox18) plants. Proteins extracted from the same tissue as used for RNA preparation were

analyzed by Western blotting and detected with anti-CCA1 antibody (8). The arrow and line indicate CCA1 and nonspecific cross-reacting proteins, respectively. Experiments were repeated twice with similar results.

Fig. 12. illustrates that CK2 can interact with and phosphorylate LHY *in vitro*. Fig. 12A shows interaction of LHY with CKB3 in vitro. ^{35}S-labeled CKB3 was mixed with GST, GST-CCA1 or GST-LHY immobilized on glutathione-agarose beads. Bound proteins were analyzed by 12.5% SDS-PAGE. The input lane represents 10% of the 35S-labeled CKB3 used and the other lanes show the amount of bound CKB3. Molecular size markers are given to the left in kiloDaltons. Fig. 12B illustrates phosphorylation of LHY by CK2 *in vitro*. GST (lane 1) or GST-LHY (lanes 2 and 3) was incubated with 280 ng of CKA1 (lanes 1 and 3) or without CKA1 (lane 2) in the presence of [γ-^{32}P]ATP. The arrow indicates the position of the GST-LHY protein after electrophoresis. Fig. 12C shows that CK2 β-subunits enhance phosphorylation of LHY by CKA1. GST alone (lanes 1 and 2) or GST-LHY (lanes 3-5) was incubated with 14 ng of CKA1 and 35 ng of CKB1 (lanes 1 and 4) or CKB3 (lanes 2 and 5) or without β-subunits (lane 3). The arrow indicates the position of the GST-LHY protein.

Fig. 13. shows that the periods of output genes are shortened in the *CKB3*-ox transgenic plants. Total RNA was isolated from wild-type and *CKB3*-ox (line ox18) plants grown as in Fig. 11, and expression of *Lhcb1*1* (Fig. 13A), *CCR2* (Fig. 13B), *CAT2* (Fig. 13C) and *CAT3* (Fig. 13D) RNAs was analyzed with ^{32}P-labeled probes. The *UBQ10* RNA levels were used as an internal control. Closed squares (solid line), wild-type; open circles, *CKB3*-ox. The bar represents the subjective light conditions for the wild-type plants. Similar results were obtained in three experiments and with another *CKB3*-ox (ox41) line.

DETAILED DESCRIPTION

OF THE PREFERRED EMBODIMENTS

The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide a new method of altering circadian rhythms and flowering in plants through the activation of CK2, particularly by overexpression of β-subunits such as the newly-discovered *CKB3*.

Standard methods of molecular biology were used in the experiments leading to the present invention. The basic methods are briefly listed below for the edification of one of skill in the art.

Yeast Strains and Expression Plasmid. *Saccharomyces cerevisiae* Y190 and pAS2, pAS-SNF1, and pACT were obtained from the Arabidopsis Biological Resource Center (ABRC). YDH8 and pJCR14 have been described (20, 45). pKT-CKB3 was constructed by ligating the *Arabidopsis CKB3* cDNA into pKT10 (58).

Yeast Two-hybrid Screen and cDNA Isolation. For the yeast two-hybrid screen, the entire coding region for *CCA1* was fused to the GAL4 DNA-binding domain (GAL4-DB) in pAS2. Y190 was transformed with the resulting plasmid, pAS-CCA1, then with a library made from *Arabidopsis* cDNAs fused to the GAL4 transactivation domain (GAL4-AD) (ABRC). Transformants ($4X10^6$) were analyzed as described previously (11). To obtain a cDNA for the full-length *CKB3* gene, an *Arabidopsis* cDNA library in λgt22 (63) was screened with the selected clone 106. DNA sequencing was done with a Sequenase kit (United States Biochemical). The GenBank database was searched using the BLAST program.

Recombinant Proteins. pGEX-CCA1 contains cDNA encoding *CCA1* cloned into pGEX-3X (Pharmacia). pET-CKA1, pET-CKA2 have been described (39). pT7-CKB1 and pT7-CKB3 contain cDNAs encoding *Arabidopsis CKB1* (10) and CKB3 in pT7-His (50). Expression and purification of glutathione-*S*-transferase (GST) and GST-CCA1 and purification of CCA1 by cleavage of GST-CCA1 with factor Xa have been described (63). CKA1 and CKA2 were produced as described (39), and were purified on a heparin-agarose column (Bio Rad). His-tagged CKB1 and CKB3 were produced in *Escherichia coli* strain BL21(DE3), and purified on Ni-NTA agarose (Qiagen). Protein concentrations were determined by the Bradford assay (Bio-Rad).

***In Vitro* Binding Assays.** CK2 subunits and GBF4 labeled with [^{35}S]methionine were synthesized by coupled transcription-translation with wheat germ extract (Promega TNT). For *in vitro* binding, 20 µl of the reactions were added to 200 µl of binding buffer [20 mM Hepes pH 7.6, 100 mM KCl, 10% glycerol, 5 mM EDTA, 0.02% NP40, 1 mM dithiothreitol (DTT), 5 mg/ml bovine serum albumin (BSA)] followed by 10 µl of glutathione-agarose beads with bound GST or GST-CCA1 and incubated at 4 °C. The beads were washed with binding buffer, then with binding buffer without BSA. Bound proteins were eluted with 1X SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and resolved by 12.5% SDS-PAGE. ^{35}S-labeled bands were detected by autoradiography, and quantitation was performed with a PhosphorImager (Molecular Dynamics).

***In Vitro* Kinase Assays.** GST-CCA1 bound to glutathione-agarose beads was resuspended in 50 µl of CK2 buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.1 mM ATP or GTP) in the presence of 5-10 µCi of [γ-^{32}P]ATP or GTP. The reaction was started by adding CK2 or whole-cell extracts (WCE) and incubating samples at 30°C for 30 min. WCE were prepared as described previously (3) except that the phosphatase inhibitor cocktail (5 mM

NH_4VO_3, 0.2 mM ammonium molybdate, 1 mM EGTA, 50 mM NaF) was added to extraction buffer. The beads were washed with phosphate-buffered saline (PBS) containing 1% Triton X-100 and resuspended in 1X SDS-PAGE sample buffer. The phosphorylated samples were separated by 10% SDS-PAGE. ^{32}P-labeled bands were detected by autoradiography and quantitated with a PhosphorImager.

Electrophoretic Mobility Shift Assays (EMSA). CCA1 was incubated with 0.1 ng of end-labeled A2 fragment of the *Arabidopsis Lhcb 1*3* gene (56) in the presence of 0.5 μg of poly(dI-dC) at 25 °C for 15 min. WCE were incubated in preincubation buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MnCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.01% Brij 35) at 30°C for 45 min, then incubated with the A2 probe in the presence of 1 μg of poly(dI-dC). The EMSA buffer and electrophoresis conditions have been described (56). DNA-protein complexes were detected by autoradiography.

The following results were obtained using the above methods.

Isolation and analysis of the *CKB3* cDNA. To isolate proteins that interact with CCA1, the yeast two-hybrid system that uses GAL4 recognition sites to regulate expression of both *HIS3* and *lacZ* genes was used (21). The GAL4-DB-CCA1 fusion protein did not itself activate transcription of the reporter genes. Four positive colonies were obtained which contained plasmid that activated *HIS3* and *lacZ* transcription only in the presence of GAL4-DB-CCA1. These fell into two classes based on sequence analysis, and one of them, clone 106, was fully characterized.

The reading frame of clone 106 encoded a 214 amino acid fragment. Because a putative translation initiation codon was missing in this cDNA clone, corresponding clones were isolated from an *Arabidopsis* cDNA library. The sequence of the full-length cDNA insert is shown in Fig. 1A. The 276 amino acid residues open reading frame encodes an estimated 30.8 kDa protein. The first ATG codon of the open reading frame starts at 142 bp and is preceded by an in-frame

stop codon at the -42 to -40 position and by a purine (A) at the -3 position. This is a favorable context for an initiation codon in plants (32). The deduced amino acid sequence is highly homologous to the β-subunit of protein kinase CK2, in particular to *Arabidopsis* CKB1 and CKB2. Thus, the gene corresponding to this cDNA clone was designated *CKB3*. The nucleic acid sequence is separately illustrated as SEQ. I.D. No. 1 in Fig. 2 while the amino acid sequence is separately illustrated as SEQ. I.D. No. 2 in Fig. 3.

Fig. 10B shows an alignment of the amino acid sequences of *Arabidopsis* CKB1, CKB2, and CKB3. The amino acid identities between CKB3 and CKB1, CKB3 and CKB2, and CKB1 and CKB2 are 75%, 71% and 80%, respectively. The similarity is greatest over the carboxyl-terminal two thirds of the three proteins. The CKB3 protein shares most of the structural features of CKB1 and CKB2 at the level of primary structure (10). First, CKB3 contains a potential metal-binding motif Cys-Pro-X_3-Cys-X_{22}-Cys-Pro-X-Cys (45) (wherein "X" may be any amino acid). Second, although the conserved autophosphorylation site, Ser-Ser-Ser-Glu-Glu, is missing in the amino-terminal region of CKB3, there are two CK2 recognition phosphorylation sites, ^{81}Ser-Gly-Ser-Glu-Gly-Asp and ^{83}Ser-Glu-Gly-Asp-Asp, in about the same location as in the animal β-subunits. Third, CKB3 has an N-terminal extension preceding the putative phosphorylation sites which exhibits a moderate level of similarity to the N-terminal extension of the other *Arabidopsis* β-subunits. Neither yeast nor animal β-subunits contain such an N-terminal extension, and this region bears no extensive similarity to other proteins.

***CKB3* has functional similarity to *CKB1* and *CKB2*.** *S. cerevisiae* has two genes coding for the catalytic (α) subunits of CK2, and at least one of the two genes is required for vegetative growth. YDH8, which carries the *cka1-Δ1 cka2-8* mutation, grows at 25 °C, but not at 35.5 °C (20), and this temperature sensitivity can be compensated for by overexpressing CK2 β-subunits, including *Arabidopsis* CKB1

and CKB2 (10). We tested whether *CKB3* could also compensate for the temperature sensitivity of the mutation. As controls, pKT10 and pJCR14, which contains the *S. cerevisiae CKB2* gene, were also transformed into YDH8. Fig. 4 shows that YDH8 cells expressing either *S. cerevisiae CKB2* or *Arabidopsis CKB3* could grow both at 25°C and 35.5°C, while transformants with pKT10 could grow only at 25°C. These results demonstrate that *CKB3* shares functional similarity with *CKB1* and *CKB2*.

CCA1 can interact with both α- and β-subunits of CK2. Although CKB1, CKB2, and CKB3 share a high degree of structural and functional homology, it might be that CKB3 is the only β–subunit that associates with CCA1 specifically. Therefore, we tested whether CKB1 and/or CKB2 are also able to interact with CCA1 in the yeast two-hybrid system and confirmed that the full-length CKB3 could interact with CCA1 in the same way as the product of the original cDNA clone. Fig. 5A demonstrates that CKB1, CKB2 and CKB3 can each interact specifically with CCA1 in yeast.

To further investigate the direct interaction of CCA1 with β–subunits of CK2, the ability of GST-CCA1 to bind to CK2 β-subunits *in vitro* was also tested. Fig. 5B shows that CKB1 and CKB2 bound to GST-CCA1 efficiently but not to GST alone (lanes 4 and 7). CKB3 also interacted with GST-CCA1, but apparently less efficiently than did CKB1 and CKB2 (lane 8). Similar analyses showed that CKA1 and CKA2, the two α–subunits of CK2 (39), also bound to GST-CCA1 (lane 9). While it is possible that CK2 β-subunits that could be present in the wheat germ extract might mediate the interaction of CCA1 with the CK2 α-subunit, our results suggest that CCA1 can interact with both CK2 α–subunits and β–subunits *in vitro*. As a negative control in these experiments, GBF4, a bZIP transcription factor (35), was used. GBF4 did not show a specific interaction with GST-CCA1 (lanes 11 and 12), confirming that the interaction of CCA1 with CK2 subunits is specific.

CK2 can stimulate binding of CCA1 to the *Lhcb1*3* promoter *in vitro*. The possible biochemical consequences of the interaction of CK2 β–subunits with CCA1 were examined. First, we tested whether DNA binding activity of CCA1 was affected by its interaction with CK2 β–subunits. As shown in Fig. 6, binding of CCA1 to the A2 fragment was stimulated specifically by CKB3 at low concentrations of CCA1 (lanes 3-7). CKB3 did not itself show any binding to the probe (lane 2). CKB1 also enhanced DNA binding activity of CCA1 in the same way (lanes 10-14).

CK2 can phosphorylate CCA1 *in vitro*. A second approach to understand the function of the CK2-CCA1 interaction was to determine whether recombinant CK2 can phosphorylate CCA1 *in vitro*. CCA1 has several putative sites for phosphorylation by CK2. We initially tested phosphorylation of CCA1 by CKA1, one of the α–subunits of CK2. Fig. 7A shows that a large amount of CKA1 (280 ng) phosphorylates GST-CCA1 but not GST (lanes 1 and 3). It has been shown that CK2 β–subunits stimulate the catalytic activity of α–subunits toward most substrates (42, 24, 1,). Fig. 7B shows that when a smaller amount (14 ng) of CKA1 was used, a strong stimulation of the CCA1 phosphorylation was observed by adding either CKB1 or CKB3 (lanes 2-4). Similar results were obtained for CKA2, the other α–subunit of CK2 (data not shown). Fig. 7B also shows that CCA1 could be phosphorylated in the presence of GTP as well as in the presence of ATP (lanes 5-7). These data confirm that the phosphorylation can be attributed to CK2 activity, because CK2 is unique among protein kinases in that it can use both ATP and GTP as a phosphodonor.

Phosphorylation by CK2 has been shown to affect the DNA binding activity of many transcription factors. Therefore, the possible effect of CK2 phosphorylation on the DNA binding activity of CCA1 was examined. When recombinant CCA1 was phosphorylated by CK2, no effect on its DNA binding activity was observed in the EMSA assay (data not shown).

***Arabidopsis* plants contain a CK2-like activity that can phosphorylate CCA1 *in vitro*.** We next examined whether plants contain a CK2-like protein kinase activity that can phosphorylate CCA1 *in vitro*. Fig. 8 shows that GST-CCA1, but not GST alone, was phosphorylated by a kinase activity in *Arabidopsis* whole-cell extracts *in vitro* (lanes 1 and 2). Fig. 8 also shows that this kinase activity was able to utilize both ATP and GTP as a phosphodonor (lane 5). Furthermore, addition of 2,3-diphosphoglycerate, which is an inhibitor of CK2 (18), reduced the incorporation of ATP into GST-CCA1 by 63% (lane 3). When this inhibitor was added to recombinant CK2, the phosphorylation of CCA1 was reduced by 77% (lane 7). These results demonstrate that the *Arabidopsis* plants contain a CK2-like activity that phosphorylates CCA1 *in vitro*, and that this kinase activity is responsible for much of the phosphorylating activity on CCA1 in the extracts.

Phosphorylation by CK2 is required for formation of the DNA-protein complex containing CCA1 in plant extracts. Fig. 9A shows that the DNA binding activity of the major CCA1-containing complex in plant extracts depends on phosphorylation. When the extracts were treated with λ protein phosphatase, formation of the complex was inhibited completely. The major DNA-protein complex containing CCA1 (marked with arrows) migrated more slowly than that formed with recombinant CCA1, suggesting that other proteins in the plant extracts are included in this complex, interacting with CCA1 and/or binding to the DNA. The A2 fragment used as a probe includes known binding sites for G-box and CAAT binding proteins (56). Fig. 9B shows that this complex is more abundant in extracts of plants expressing CCA1 under the control of a constitutive promoter (62). Further evidence that the marked complex contains CCA1 includes the observations (data not shown) that this complex is absent in extracts prepared from CCA1-null mutant plants and that addition of anti-CCA1 antibodies inhibits its formation. Fig. 9C demonstrates that the CK2-like phosphorylation activity in the extracts is important for this binding activity. Formation of the complex was

abolished when CK2 inhibitors, either 2,3-diphosphoglycerate or quercetin, were added to the preincubation reactions. 2,3-diphosphoglycerate inhibits both CK1 and CK2, whereas inhibition by quercetin is specific to CK2 (18, 9). The components of the new bands that appear in the inhibitor treated samples are not yet known, but these bands presumably represent a change in the composition of the CCA1-containing complex in the absence of CK2-mediated phosphorylation. Taken together, these results demonstrate that phosphorylation by CK2 is required in the plant extracts for formation of the major DNA-protein complex containing CCA1.

The protein kinase CK2 is a Ser/Thr kinase that is ubiquitously expressed and highly conserved (42, 24, 1). CK2 consists of two catalytic (α) and two regulatory (β) subunits, which form an $\alpha_2\beta_2$ heteromeric holoenzyme. Although most organisms have two genes encoding α-subunits and one gene encoding the β-subunit, two genes encoding β-subunits have been reported in *S. cerevisiae* and *Arabidopsis* (45, 10, 3). The CKB3 protein exhibits significant amino acid sequence identity with *Arabidopsis* CKB1 and CKB2. Several lines of evidence confirm that CKB3 does indeed function as a third CK2 β-subunit in *Arabidopsis*. First, CKB3 was able to compensate for the temperature-sensitive growth defect of an *S. cerevisiae cka1-Δ1 cka2-8* mutation. Second, recombinant CKB3 was able to stimulate the catalytic activity of CKA1 when CCA1 was used as a substrate. CKB3 is the only reported example of a third CK2 β-subunit in any organism. Interestingly, it had been suggested previously that there might be a third CK2 α-subunit in *Arabidopsis* (39). It is yet to be determined whether there are several forms of the holoenzyme with different subunit compositions or whether different subunits confer different substrate specificities and/or tissue specificities.

We have shown that CK2 β-subunits specifically interact with CCA1 both in yeast and in an *in vitro* interaction assay. We did not isolate clones for the two other CK2 β-subunits in the initial screen, but we have observed that the growth of yeast

containing the constructs for these subunits is slower than that of cells expressing the CAL4-AD-CKB3 construct, and this may account for our failure to identify them along with CKB3.

We have found that recombinant His-tagged CK2 β–subunits stimulate binding of CCA1 to a fragment of the *Lhcb1*3* gene. This effect is likely to be specific because OBP1, a DNA binding protein that stimulates interaction of OBF4 and OBF5 with ocs elements, did not affect binding of CCA1 to the A2 fragment (66). Also, both recombinant GST-CKB1 and GST-CKB3 stimulated DNA binding of CCA1, whereas GST alone had no effect (data not shown), demonstrating that CK2 β–subunits are responsible for the enhancement. The fact that the mobility of the complex was not affected suggests that the interaction of the proteins might be transient or unstable under the conditions for the EMSA. There have been other such reports of enhancement of DNA binding by a second protein without altering the mobility of the DNA-protein complex (66, 60, 31, 11).

The fact that CK2 β–subunits associate with CCA1 and stimulate its binding to the *Lhcb1*3* promoter suggests a different mechanism for regulation of CCA1 DNA binding activity other than phosphorylation. In fact, the α–subunit is not required for this stimulation, and CK2 β–subunits cannot themselves phosphorylate CCA1. It is possible that in addition to being the regulatory subunit of CK2, the β-subunit might play other roles in the cell. Overexpression of the CK2 β–subunit in *Schizosaccharomyces pombe* causes multiple septation and inhibits cell growth and cytokinesis (47). These phenotypes appeared to be due to the production of free β-subunit rather than to excess holoenzyme. In *Xenopus* oocytes, the β-subunit interacts with Mos, a germ cell-specific Ser/Thr kinase that is required for oocyte maturation, and this interaction negatively regulates Mos-mediated mitogen-activated protein kinase activation resulting in repression of oocyte maturation (7, 6). Recently, it was also shown that cyclin D, which is a regulatory component of

complexes of cyclin with cyclin dependent kinase (Cdk), stimulates transcriptional activity of estrogen receptor independent of interaction with Cdks (67). Therefore, it is intriguing to speculate that direct interaction of CK2 β–subunits with CCA1 stimulates binding of CCA1 to promoter sequences and can affect CCA1-mediated transcription.

We have demonstrated that CKA1 phosphorylates CCA1 *in vitro* and that both CKB1 and CKB3 stimulate this phosphorylation. Although CKA1CKB1 showed a higher activity of CCA1 phosphorylation than CKA1CKB3, the possibility that this was due to differing relative activities of CKB1 and CKB3 in their corresponding preparations cannot be excluded. We have also demonstrated that *Arabidopsis* plants contain a CK2-like protein kinase activity that can phosphorylate CCA1 *in vitro*, and that this is a major kinase activity for CCA1 phosphorylation in the extracts. The identity of this kinase was confirmed in two ways. First, the kinase activity phosphorylates CCA1 in the presence of GTP as well as ATP, a unique characteristic that distinguishes CK2 from other Ser/Thr kinases. Second, addition of 2,3,-diphosphoglycerate, an inhibitor of CK2, inhibited most of the CCA1 phosphorylating activity in the plant extracts.

Phosphorylation of transcription factors by CK2 has been reported to modulate their DNA binding activity, cellular localization, metabolism, and interaction with other proteins (1, 12, 28, 40, 2, 25, 34, 41). It was recently shown that the *Lhcb1*1* RNA level in transgenic plants overexpressing CCA1 decreased steadily when plants were transferred from light-dark cycles into constant dark even though CCA1 was expressed at a high level (62). We have also observed that the *Lhcb1*1* RNA level in etiolated transgenic plants overexpressing CCA1 was as low as that in etiolated wild type plants (data not shown). These observations suggest that CCA1 activity is regulated by light through posttranslational modifications, one of which could be phosphorylation. In this regard, our finding that plant extracts contain a CK2-like activity that is required for formation of the major DNA-protein

complex containing CCA1 is especially noteworthy. The CCA1-containing complex is likely to contain a protein or proteins in addition to CCA1. Although CK2 phosphorylation of recombinant CCA1 did not affect its DNA binding activity *in vitro*, it is possible that in the plants the other proteins affect the relative binding affinities of the phosphorylated and non-phosphorylated forms of CCA1 for its binding sites. Alternatively, the phosphorylation state of CCA1 might be important for protein-protein interactions of CCA1 with other protein(s) in the complex. It is also possible that phosphorylation of other protein(s) by CK2 is essential for the CCA1 complex formation.

Overexpression of *CKB3* To further explore the hypothesis that the CK2/CCA1 DNA-protein complex plays a role in the regulation of the circadian clock, we created transgenic *Arabidopsis* plants overexpressing a c-myc tagged form of *CKB3* and analyzed their circadian behavior. To produce the tagged *CKB3 an* Eco RI-Bsr GI fragment of the plasmid pUC-CKB3 that contains the entire coding sequence of *CKB3* cDNA at the Bam HI site of pUC19 was replaced with the duplex DNA composed of oligonucleotide myc-CKB3F (5'-AATTGAGATCTCATGGAGCAAAAGCTTATC AGCGAGGAGGACTTGAACAT) and oligo-nucleotide myc-CKB3B (5'-GTACATGTTCAAGTCCTCCTCGCTGATAAGCTTTTGCTCCATGAGATCT) to introduce the Bgl II site and c-myc encoding sequence in front of *CKB3*. The resultant plasmid was digested with Bgl II and Hinc II, and the Bgl II-Hinc II fragment was subcloned into the pBI121 vector (Clonetech). This construct was used to transform *Agrobacterium tumefaciens* strain A2260, and then *Arabidopsis* plants (Columbia ecotype) using the *in planta* transformation procedure as described (61, 62). Overexpression of *CKB3* had no apparent effects on plant growth and development except timing of flowering.

From 16 transgenic lines that each had a single site of insertion, two transgenic lines designated ox18 and ox41 were further analyzed. Levels of *CKB3*

transcript in the fourth generation of homozygous *CKB3*-overexpressing (*CKB3*-ox) plants were approximately 20 times higher than that in the wild-type (Fig. 10A). Ten μg of total RNA were treated with RQ1 RNase-free DNase (Promega) and the first-strand cDNA was synthesized as described in (43) The product of the first-strand synthesis was then used for PCR to amplify 140 bp *CKB3* cDNA with the primers CKB3F1 (5'-ACAAGGAACGTAGTGGAGGAGGTG) and CKB3B3 (5'-AACCCTAGATGT GGTGGTGGAAG). As a control, primers UBQ10-5' and UBQ10-3' (61, 62) were used to amplify 111 bp *UBQ10* cDNA. The resultant PCR fragments were separated on a 2% agarose gel, blotted and hybridized with ^{32}P-labeled probes.

The transgenic plants contained appreciable amounts of the c-myc-tagged CKB3 protein (Fig. 10B). Protein extracts were obtained by grinding 10-day-old seedlings in 100 μl of 3XSDS-sample buffer (180 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 7.5% 2-mercaptoethanol), boiling this mixture for 5 min and saving the supernatant after centrifugation for 15 min at 14,000x*g*. Protein concentration was measured with a protein assay reagent (Bio-Rad). Western blots were performed using anti-c-myc monoclonal antibody 9E10 (17, 15, 64, 46) following the methods described in (61, 62).Measurement of CK2 activity in whole-cell extracts showed that the transgenic lines exhibited a 1.7-fold increase in CK2 activity (Fig. 10C). Frozen seedlings (100 mg) were ground and extracted with 100 μl of extraction buffer (50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 0.1 M KCl, 0.25 M sucrose and 10% glycerol, 1 mM phenylmethylsulfonylfluoride, protease inhibitor cocktail (Boehringer Mannheim), phosphatase inhibitor mixture (55, 26, 48) and 14 mM 2-mercaptoethanol). After centrifugation at 14,000x*g* for 15 min, the supernatant was saved and protein concentration was measured as above. CK2 assays were carried out at 37 °C with 200 μM CK2 specific peptide substrate Arg-Arg-Arg-Asp-Asp-Asp-Ser-Asp-Asp-Asp (Boehringer Mannheim) in 25 μl of CK2 buffer (55, 26, 48) as described (5).

We next examined whether *CKB3* overexpression affected circadian expression of the *CCA1* and *LHY* genes. Oscillations in expression of these genes are robust in wild-type plants transferred into continuous light. Figs. 11A and 11B show that the periods of *CCA1* and *LHY* RNAs were shortened by about four hours in the *CKB3*-ox plants (total RNA isolation and RNA blot analyses were performed as described in (61, 62, 19)), and no differences in the amplitude of the rhythms were seen. Fig. 11C shows that the period of the CCA1 protein oscillation was also shortened (immunoblot analyses of the CCA1 protein were carried out as explained in (61, 62)). Thus, overexpression of *CKB3* affected circadian rhythms of these two genes that are closely associated with the circadian clock.

Because LHY is closely related to CCA1 both structurally and functionally (49), we tested the possibility that CK2 can also interact with and phosphorylate LHY. Fig. 12A shows that, like CCA1, LHY could bind to CKB1 and CKB3 efficiently and specifically. To make this determination a PCR fragment containing the entire *LHY* gene was cloned in pGEX-3X (Pharmacia). The GST-LHY fusion protein was produced and purified as described in (55, 26, 48). *In vitro* binding assays were performed using ^{35}S-labeled CKB3 and glutathione-agarose beads (Sigma) with bound GST or GST-LHY. Furthermore, Fig. 12B shows that CKA1, the α-subunit of CK2, could phosphorylate LHY, and this phosphorylation was enhanced by adding either CKB1 or CKB3. *In vitro* kinase assays were performed using GST-LHY bound to glutathione-agarose beads and recombinant CK2α- and β-subunits as described in (55, 26, 48). These data are consistent with the idea that CK2 can interact with and phosphorylate both CCA1 and LHY in *Arabidopsis*.

If the function of both CCA1 and LHY is closely associated with a central oscillator and this is altered by increased CK2 activity, then the period lengths of the circadian rhythms of output genes should also be changed to reflect that of the *CCA1* and *LHY* RNA rhythms. We therefore tested whether overexpression of *CKB3* affected genes representing different rhythmic outputs of the circadian clock.

*Lhcb1*1* and *CAT2* RNAs normally peak around subjective dawn and during the subjective day, whereas *CAT3* and *CCR2* RNAs peak considerably later in wild-type plants. Fig. 13 shows that overexpression of *CKB3* had the same effect on the circadian expression of these genes as it did on the *CCA1* and *LHY* RNA rhythms. Although it did not alter the amplitudes, it shortened the periods of the rhythms by about four hours.

It has been shown that the circadian clock is involved in the control of hypocotyl elongation and the photoperiodic flowering response (13). Overexpression of *CKB3* did not affect hypocotyl elongation under continuous white light or dark conditions. Hypocotyl lengths of 5-day-old seedlings were measured using a digital camera (Kodak DCS 420) and the NIH Image program. The average hypocotyl lengths of plants grown in continuous white light were: wild-type, 2.19±0.09 mm; *CKB3*-ox, 2.23±0.14 mm (line ox18) and 2.06±0.85 mm (line ox41); in the dark: wild-type, 11.6±0.28; *CKB3*-ox, 11.3±0.30 mm (line ox18) and 11.6±0.28 mm (line ox41). However, *CKB3* overexpression did affect the photoperiodic induction of flowering. Table 1 shows that *CKB3*-ox lines flowered earlier than wild-type in short-day conditions, whereas *CKB3* overexpression did not substantially affect flowering time in long-day conditions. To obtain data for the table plants were grown under long-day (L:D 16:8) or short-day (L:D 8:16) conditions as described in (20). The table shows the number of total leaves (including cauline leaves on the main stem) on the day when the first flower opened. Leaves of 10-20 plants were counted and values reported are means ±Standard Error. Experiments were done three times under long-day and twice under short-day conditions with similar results. Under long-day conditions both the experimental and wild type plants have nearly the same number of leaves showing that flowering response was essentially unaltered. However, under short-day conditions the wild type plants take much longer to flower growing significantly larger (more leaves) than the experimental plants. Thus, *CKB3* overexpression caused a diminished photoperiodic flowering

response, and while we cannot rule out other possibilities, this effect may well be a result of the altered clock function in *CKB3*-ox plants.

Table 1

	Long Days	Short Days
Wild Type	14.2 ± 0.2	50.3 ± 1.6
CKB3-ox18	12.1 ±0.2	28.1 ± 1.0
CKB3-ox41	12.0 ±0.2	26.9 ± 0.9

These data show that an increase in CK2 activity alters circadian rhythms in *Arabidopsis*. CK2 can interact with and phosphorylate both CCA1 and LHY, proteins that are closely associated with the central oscillator of the circadian clock. Overexpression of *CKB3* substantially increased the catalytic activity of CK2 in the plant in a way similar to that seen in other organisms (47). This overexpression in *Arabidopsis* caused shorter periods of circadian oscillations of both *CCA1* and *LHY* RNAs as well as those of several output genes. It also affected the timing of flowering, but did not affect hypocotyl elongation. In these respects, the CKB3-ox lines are similar to phenotypes of *toc1*, as both lines exhibited shorter periods of output genes, a reduced photoperiodic flowering response in the Columbia ecotype, but normal hypocotyl elongation under light and dark conditions (37, 52). Two classes of mutants which affect the input pathway, *det1* and plants overexpressing *phyA* or *phyB*, also exhibited a short-period phenotype (4, 8, 38, 53). However, morphological phenotypes that are also associated with these lines are absent in the *CKB3*-ox and *toc1* plants. Furthermore, in contrast to the higher *Lhcb1*1* expression seen in the *det1* and phyA/B-overexpressing lines, induction of *Lhcb1*1* in *CKB3*-ox plants by brief red illumination was reduced compared to wild-type (data not shown). This observation is consistent with the finding that antisense expression of the CK2 α-subunit gene increased the expression of *Lhcb1*1* under

similar conditions (30). We conclude that CK2 affects components that are part of the central oscillator itself or closely associated with it.

Recently, mutant alleles of a clock gene, *double-time* (*dbt*), were isolated in *Drosophila* (29). These mutations alter the periods of behavioral rhythms and molecular oscillations of clock components PER and TIM and also affect PER phosphorylation and stability. The *dbt* gene encodes a protein (DBT) closely related to human casein kinase Iε (29). Furthermore, DBT can interact with PER *in vitro* and in *Drosophila* cells, suggesting that DBT regulates PER phosphorylation directly. Both casein kinase Iε and CK2 are Ser/Thr kinases that do not require a second messenger as a cofactor, and both prefer acidic substrates such as casein (42, 54, 24). Our finding that CK2 can interact with and phosphorylate both CCA1 and LHY *in vitro* is consistent with the idea that CK2 could modulate the circadian clock by direct interaction with and/or phosphorylation of the CCA1 and LHY proteins. Such interactions may control the activity and/or stability of these proteins. We have found that CCA1 and LHY can interact *in vitro* (data not shown) and such an interaction could also be a target for modification by CK2. Because the periods of *LHY* and several output genes expression were also shortened in a *CCA1*-null mutant line (19), we suggest that *CKB3* overexpression might inactivate and/or destabilize CCA1 and LHY by altering their phosphorylation state. We cannot exclude the possibility that CKB3 affects clock components other than or in addition to CCA1 and LHY. However, the results presented here clearly demonstrate that the protein kinase CK2 is involved in the function of the circadian clock in *Arabidopsis*.

In summary, interaction of subunits of CK2 with CCA1 and phosphorylation of CCA1 by CK2 may modulate CCA1 activities that are required for phytochrome regulation of *Lhcb1*3* gene expression and for circadian clock function. In light of the involvement of CCA1 in circadian rhythms (62), it is of particular interest that the clock gene affected in the *double-time* mutant of *Drosophila* and which is required for circadian rhythmicity has recently been cloned and found to be closely

related to human casein kinase Iε (29). While the physiological significance of the CK2-CCA1 association remains to be elucidated, our findings should be important steps toward understanding the regulation of CCA1 function in *Arabidopsis*.

In addition to the equivalents of the claimed elements, obvious substitutions now or later known to one with ordinary skill in the art are defined to be within the scope of the defined elements. The claims are thus to be understood to include what is specifically illustrated and described above, what is conceptually equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention. Those skilled in the art will appreciate that various adaptations and modifications of the just-described preferred embodiment can be configured without departing from the scope and spirit of the invention. The illustrated embodiment has been set forth only for the purposes of example and that should not be taken as limiting the invention. Therefore, it is to be understood that, within the scope of the appended claims, the invention may be practiced other than as specifically described herein.

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